PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 :
C12N 5/14, A01H 5/00
C12N 15/82, 9/00

A1

(11) International Publication Number: **WO 92/01042**

(43) International Publication Date: 23 January 1992 (23.01.92)

(21) International Application Number: PCT/DK91/00194

(22) International Filing Date: 5 July 1991 (05.07.91)

(30) Priority data:
1627/90 6 July 1990 (06.07.90) DK

(71) Applicant *(for all designated States except US)*: NOVO NORDISK A/S [DK/DK]; Novo Allé, DK-2880 Bagsvaerd (DK).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)* : WILLMITZER, Lothar [DE/DE]; Am Kleinen Wannsee 34, D-1000 Berlin 39 (DE). SONNEWALD, Uwe [DE/DE]; Gierkezeile 34, D-1000 Berlin 10 (DE). RÖBER, Manuela [DE/DE]; Lynarstrasse 5/2309, D-1000 Berlin 65 (DE). CARLSEN, Søren, Knud [DK/DK]; Stenhøjgaardsvej 27, DK-3460 Birkerød (DK).

(74) Common Representative: NOVO NORDISK A/S; Patent Department, Novo Allé, DK-2880 Bagsvaerd (DK).

(81) Designated States: AT (European patent), AU, BE (European patent), CA, CH (European patent), DE (European patent), DK (European patent), ES (European patent), FI, FR (European patent), GB (European patent), GR (European patent), IT (European patent), JP, LU (European patent), NL (European patent), SE (European patent), US.

Published
With international search report.

(54) Title: TRANSGENIC PLANTS EXPRESSING INDUSTRIAL ENZYMES

(57) Abstract

A transgenic plant comprises an inserted DNA sequence encoding an industrial enzyme which is heterologous to said plant, with the exception of enzymes conferring improved growth properties or desirable physical characteristics to living plants producing them. The transgenic plant may be used for the production of industrial enzymes by cultivating a seed or other propagatable part thereof, and the enzyme may then be recovered from the plant.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	ES	Spain	MG	Madagascar
AU	Australia	FI	Finland	ML	Mali
BB	Barbados	FR	France	MN	Mongolia
BE	Belgium	GA	Gabon	MR	Mauritania
BF	Burkina Faso	GB	United Kingdom	MW	Malawi
BG	Bulgaria	GN	Guinea	NL	Netherlands
BJ	Benin	GR	Greece	NO	Norway
BR	Brazil	HU	Hungary	PL	Poland
CA	Canada	IT	Italy	RO	Romania
CF	Central African Republic	JP	Japan	SD	Sudan
CG	Congo	KP	Democratic People's Republic of Korea	SE	Sweden
CH	Switzerland			SN	Senegal
CI	Côte d'Ivoire	KR	Republic of Korea	SU	Soviet Union
CM	Cameroon	LI	Liechtenstein	TD	Chad
CS	Czechoslovakia	LK	Sri Lanka	TG	Togo
DE	Germany	LU	Luxembourg	US	United States of America
DK	Denmark	MC	Monaco		

TRANSGENIC PLANTS EXPRESSING INDUSTRIAL ENZYMES

FIELD OF INVENTION

The present invention relates to plant cells and transgenic plants containing inserted DNA sequences encoding industrial enzymes, as well as to a method of producing industrial enzymes by growing a plant containing such a DNA sequence.

BACKGROUND OF THE INVENTION

Industrial enzymes are enzymes which are used in industrial processes or which are components of industrial products. Thus, for instance proteases may be used in the detergent, textile, food or tanning industries, cellulases may be used in the detergent, paper, textile, feed or sugar industry as well as in the production of beer, juice or wine, amylases may be used for baking or brewing as well as in the starch and sugar industries, lipases may be used in detergents as well as in the synthesis of lipids or other processes of organic synthesis, phytases, phosphatases and pectinases may be used in the feed industry, xylanases, mannanases and ligninases in the pulp and paper industry, peroxidases in the detergent and textile industry etc. The use of these and other enzymes for industrial purposes is widespread and well documented in prior literature.

The technology most widely used at present for the production of industrial enzymes utilizes the fact that microorganisms such as fungi and bacteria are able to produce large amounts of certain proteins. A number of such microorganisms have been improved by either classical genetic means, i.e. mutation and selection or by recombinant DNA methods resulting in the development of rather efficient strains for the production of industrial enzymes.

The use of this technology may be limited in certain respects. First of all, certain industrial enzymes may need post-

translational modifications in order to obtain full enzymatic activity. This may be in the form of glycosylation, phosphorylation or processing of a mature protein from a pre-pro form. In several cases these posttranslational modifications either do not take place at all or take place with a rather low efficiency in microorganisms. Thus the production of industrial enzymes in higher eucaryotic cells which are able to perform these modifications might be advantageous. Secondly, the production of industrial enzymes in microorganisms usually requires the use of large fermenters to grow the microorganisms. Running a fermenter implies high energy requirements, use of special chemicals for preparing the growth media and waste-water problems, all of which add up to a very cost-intensive process.

SUMMARY OF THE INVENTION

The present inventors have developed an alternative process for the production of industrial enzymes which eliminates the two major problems indicated above. By introducing DNA sequences coding for industrial enzymes into plant cells, regenerating transgenic plants from such cells and growing the transgenic plants, it has been found possible to produce industrial enzymes which exhibit the desired activity.

Accordingly, the present invention relates to a plant cell which comprises an inserted DNA sequence encoding an industrial enzyme which is heterologous to said plant cell, with the exception of enzymes conferring improved growth properties or desirable physical characteristics to living plants producing them.

It has previously been described to introduce DNA sequences into plants, which sequences code for protein products, including enzymes, imparting to the transformed plants certain desirable properties such as increased resistance against pests, pathogens, herbicides or stress conditions (cf. for

instance EP 90 033, EP 131 620, EP 205 518, EP 270 355, WO 89/04371 or WO 90/02804), or an improved nutrient value of the plant proteins (cf. for instance EP 90 033, EP 205 518 or WO 89/04371). Furthermore, WO 89/12386 discloses the transformation of plant cells with a gene coding for levansucrase or dextransucrase, regeneration of the plant (especially a tomato plant) from the cell resulting in fruit products with altered viscosity characteristics.

It should be noted that, according to the present invention, the DNA sequence encoding the industrial enzyme is intoduced into the plant cell with the sole purpose of utilizing the regenerated transgenic plant as a production organism which is able to generate the enzyme in the active form and from which the enzyme may then be recovered, if desired. Consequently, the enzyme will typically not confer improved growth properties (e.g. increased resistance against pests or pathogens), a higher content of nutrients (by means of an altered amino acid composition) or desirable physical characteristics (e.g reduced viscosity of fruit products) to the plant (if it does, this will be incidental to the true purpose which is to synthesize the enzyme).

In another aspect, the present invention relates to a transgenic plant which comprises an inserted DNA sequence encoding an industrial enzyme which is heterologous to said plant, with the exception of enzymes conferring improved growth properties or desirable physical characteristics to living plants producing them.

In the present context, the term "heterologous" is understood to indicate that the enzyme in question is not produced by the plant species in nature.

In a further aspect, the present invention relates to a method of producing an industrial enzyme in a plant, the method comprising cultivating a plant according to the invention or a

seed or other propagatable part thereof under conditions conducive to the production of the enzyme, and, if desired, recovering the enzyme from the plant.

In the present context, the term "other propagatable part" is intended to include multicellular explants (e.g. leaf discs, stem segments, roots, etc.), somatic embryos or other plant parts which may be propagated into whole plants.

The production of industrial enzymes in higher plants overcomes the two major obstacles to the production of industrial enzymes in microorganisms indicated above. Firstly, higher plants have the biosynthetic capacity to perform the requisite post-translational modifications occuring in eucaryotic cells of mammalian or other origin. Secondly, transgenic plants grown in the field need very little extra energy for growth (and hence for the production of proteins such as industrial enzymes) and furthermore do not give rise to any major problems with respect to waste management.

DETAILED DISCLOSURE OF THE INVENTION

In the plant cell according to the invention, the DNA sequence encoding the enzyme is under the control of a regulatory sequence which directs the expression of the enzyme from the DNA sequence in plant cells and intact plants. The regulatory sequence may be either endogenous or heterologous to the host plant cell.

The regulatory sequence may comprise a promoter capable of directing the transcription of the DNA sequence encoding the enzyme in plants. Examples of promoters which may be used according to the invention are the 35s RNA promoter from cauliflower mosaic virus (CaMV), the class I patatin gene B 33 promoter, the ST-LS1 gene promoter, promoters conferring seed-specific expression, e.g. the phaseolin promoter, or promoters which are activated on wounding, such as the promoter of the

proteinase inhibitor II gene or the wun1 or wun2 genes. In a particular embodiment, wherein the promoter may be operably connected to an enhancer sequence, the purpose of which is to ensure increased transcription of the DNA sequence encoding the enzyme. Examples of useful enhancer sequences are enhancers from the 5'-upstream region of the 35s RNA of CaMV, the 5'-upstream region of the ST-LS1 gene, the 5'-upstream region of the Cab gene from wheat, the 5'-upstream region of the 1'- and 2'-genes of the T_R-DNA of the Ti plasmid pTi ACH5, the 5'-upstream region of the octopine synthase gene, the 5'-upstream region of the leghemoglobin gene, etc.

The regulatory sequence may also comprise a terminator capable of terminating the transcription of the DNA sequence encoding the enzyme in plants. Examples of suitable terminators are the terminator of the octopine synthase gene of the T-DNA of the Ti-plasmid pTiACH5 of Agrobacterium tumefaciens, of the gene 7 of the T-DNA of the Ti plasmid pTiACH5, of the nopaline synthase gene, of the 35s RNA-coding gene from CaMV or from various plant genes, e.g. the ST-LS1 gene, the Cab gene from wheat, class I and class II patatin genes, etc.

In another embodiment, the DNA sequence encoding the enzyme may be operably connected to a DNA sequence encoding a leader peptide capable of directing the transport of the expressed enzyme to a specific cellular compartment (e.g. vacuoles) or to extracellular space. Examples of suitable leader peptides are the leader peptide of proteinase inhibitor II from potato, the leader peptide and an additional about 100 amino acid fragments of patatin, or the transit peptide of various nucleus-encoded proteins directed into chloroplasts (e.g. from the St-LS1 gene, SS-Rubisco genes, etc.) or into mitochondria (e.g. from the ADP/ATP translocator).

In a further embodiment, the DNA sequence encoding the enzyme may be modified in the 5' non-translated region resulting in enhanced translation of the sequence. Such modifications may,

for instance, result in removal of hairpin loops in RNA of the 5' non-translated region. According to the invention, translation enhancement may be provided by suitably modifying the omega sequence of tobacco mosaic virus or the leaders of other plant viruses (e.g. BMV, MSV) or of plant genes expressed at high levels (e.g. SS-Rubisco, class I patatin or proteinase inhibitor II genes from potato).

In a still further embodiment, the DNA sequence encoding the enzyme is connected to a second DNA sequence encoding a protein or a fragment thereof in such a way that expression of said DNA sequences results in the production of a fusion protein. When the host cell is a potato plant cell, the second DNA sequence may, for instance, encode patatin or a fragment thereof (such as a fragment of about 100 amino acids).

The industrial enzyme produced by the plant may be any enzyme which may advantageously be produced by the method of the invention. Thus, the enzyme may be selected from the group consisting of proteases (e.g. aspartic proteases, serine proteases, sulfhydryl proteases, subtilisins, etc.), cellulases, hemicellulases (e.g. xylanases or mannanases), amylases, lipases, peroxidases (including ligninases), phosphatases (including phytases), oxidoreductases, glucose isomerase and pectinases.

A particularly preferred class of enzymes producible by the method of the invention is heme enzymes, i.e. enzymes containing heme (e.g. protoporphyrin IX) as a prosthetic group. The heme enzyme is preferably an oxidoreductase, in particular a peroxidase, including a lignin peroxidase or Mn-peroxidase, or haloperoxidase. Examples of suitable peroxidases are Coprinus sp. peroxidase or plant peroxidases, such as barley or horseradish peroxidase.

It is currently assumed that the present method may advantageously be used to produce enzymes derived from higher

eukaryotic organisms. Examples of such enzymes are chymosin, papain, chymopapain, trypsin (of mammalian, e.g. bovine or porcine origin) and chymotrypsin.

Examples of other enzymes which may favourably be prepared by the present process are Mucor miehei aspartic protease, Humicola, Fusarium or Bacillus cellulases, xylanases or mannanases, and feed enzymes. In the latter case, it may be advantageous to introduce the DNA sequence coding for the enzyme into a plant which is used for feed (either the entire plant or parts thereof, e.g. fruit, roots, etc.) so that it may not be required to isolate the enzyme from the plant. Examples of feed enzymes are enzymes imparting an improved digestibility to the feed, such as cellulases, hemicellulases, proteases or lipases, or which improve the nutritive value of the feed, such as phytases which provide an increased uptake of phosphate from the feed.

The plant in which the DNA sequence coding for the enzyme may suitably be a dicotyledonous plant, examples of which are is a tobacco, potato, tomato, or leguminous (e.g. bean, pea, soy, alfalfa) plant. It is, however, contemplated that monocotyledonous plants, e.g. cereals, may equally well be transformed with the DNA sequence coding for the enzyme.

Procedures for the genetic manipulation of monocotyledonous and dicotyledonous plants are well known. In order to construct foreign genes for their subsequent introduction into higher plants, numerous cloning vectors are available which generally contain a replication system for E. coli and a selectable/-screenable marker system permitting the recognition of transformed cells. These vectors include e.g. pBR322, the pUC series, pACYC, M13 mp series etc. The foreign sequence may be cloned into appropriate restriction sites. The recombinant plasmid obtained in this way may subsequently be used for the transformation of E. coli. Transformed E. coli cells may be grown in an appropriate medium, harvested and lysed. The

chimeric plasmid may then be reisolated and analyzed. Analysis of the recombinant plasmid may be performed by e.g. determination of the nucleotide sequence, restriction analysis, electrophoresis and other molecular-biochemical methods. After each manipulation the sequence may be cleaved and ligated to another DNA sequence. Each DNA-sequence can be cloned on a separate plasmid DNA. Depending on the way used for transferring the foreign DNA into plant cells other DNA sequences might be of importance. In case the Ti-plasmid or the Ri plasmid of Agrobacterium tumefaciens or Agrobacterium rhizogenes, at least the right border of the T-DNA may be used, and often both the right and the left borders of the T-DNA of the Ri or Ti plasmid will be present flanking the DNA sequence to be transferred into plant cells.

The use of the T-DNA for transferring foreign DNA into plant cells has been described extensively in the prior literature (cf. Gasser and Fraley, 1989, Science 244, 1293 - 1299 and references cited therein). After integration of the foreign DNA into the plant genome, this sequence is fairly stable at the original locus and is usually not lost in subsequent mitotic or meiotic divisions. As a general rule, a selectable marker gene will be cotransferred in addition to the gene to be transferred, which marker renders the plant cell resistant to certain antibiotics, e.g. kanamycin, hygromycin, G418 etc. This marker permits the recognition of the transformed cells containing the DNA sequence to be transferred compared to nontransformed cells.

Numerous techniques are available for the introduction of DNA into a plant cell. Examples are the Agrobacterium mediated transfer, the fusion of protoplasts with liposomes containing the respective DNA, microinjection of foreign DNA, electroporation etc. In case Agrobacterium mediated gene transfer is employed, the DNA to be transferred has to be present in special plasmids which are either of the intermediate type or the binary type. Due to the presence of sequences homologous to

T-DNA sequences, intermediate vectors may integrate into the Ri- or Ti-plasmid by homologous recombination. The Ri- or Ti-plasmid additionally contains the vir-region which is necessary for the transfer of the foreign gene into plant cells. Intermediate vectors cannot replicate in Agrobacterium species and are transferred into Agrobacterium by either direct transformation or mobilization by means of helper plasmids (conjugation). (Cf. Gasser and Fraley, op. cit. and references cited therein).

Binary vectors may replicate in both Agrobacterium species and E. coli. They may contain a selectable marker and a poly-linker region which to the left and right contains the border sequences of the T-DNA of Agrobacterium rhizogenes or Agrobacterium tumefaciens. Such vectors may be transformed directly into Agrobacterium species. The Agrobacterium cell serving as the host cell has to contain a vir-region on another plasmid. Additional T-DNA sequences may also be contained in the Agrobacterium cell.

The Agrobacterium cell containing the DNA sequences to be transferred into plant cells either on a binary vector or in the form of a cointegrate between the intermediate vector and the T-DNA region may then be used for transforming plant cells. Usually either multicellular explants (e.g. leaf discs, stem segments, roots), single cells (protoplasts) or cell suspensions are cocultivated with Agrobacterium cells containing the DNA sequence to be transferred into plant cells. The plant cells treated with the Agrobacterium cells are then selected for the cotransferred resistance marker (e.g. kanamycin) and subsequently regenerated to intact plants. These regenerated plants will then be tested for the presence of the DNA sequences to be transferred.

If the DNA is transferred by e.g. electroporation or microinjection, no special requirements are needed to effect transformation. Simple plasmids e.g. of the pUC series may be used to transform plant cells. Regenerated transgenic plants

may be grown normally in a greenhouse or under other conditions. They should display a new phenotype (e.g. production of new proteins) due to the transfer of the foreign gene(s). The transgenic plants may be crossed with other plants which may either be wild-type or transgenic plants transformed with the same or another DNA sequence. Seeds obtained from transgenic plants should be tested to assure that the new genetic trait is inherited in a stable Mendelian fashion.

GENERAL METHODS

In the following section techniques which are known per se will be described in further detail to ensure a better understanding of the examples described below.

1. Cloning procedures

Most of the cloning steps were performed in pUC 18/19 (Yanisch-Perron et al., Gene 33, 103-119) or in pMPK 110 (Eckes, Dissertation 1984, Universität zu Köln). The binary vector BIN 19 (Bevan (1984) Nucleic Acids Res. 12, 8711-8720) was used as a vector for transferring genes into plants using Agrobacterium tumefaciens.

2. Bacterial strains

For propagation of the pUC vectors, E. coli strains BMH 71-18 (Messing et al., Proc. Natl. Acad. Sci. USA 24, 6342-6346) or TB1 were used. For the vector BIN 19 only TB1 was used. TB1 is a recombination-deficient tetracycline resistant derivative of the strain JM 101 (Yanisch-Perron et al., Gene 33, 103-119).

For transformation of plant cells the binary vector BIN 19 and its derivatives were transferred into the Agrobacterium tumefaciens strain LBA 4404 (Bevan, Nucleic Acids Res. 12, 8711-8721).

3. Transformation of Agrobacterium tumefaciens

BIN 19 derivatives were transferred into Agrobacterium tumefaciens via direct transformation (Holsters et al., (1978) Mol. Gen. Genetics 163, 181-187). Plasmid DNA was isolated from Agrobacterium strains according to Birnboim and Doly (Nucleic Acids res. 7, 1513-1523) and analyzed by gel electrophoresis after digestion with restriction enzymes.

4. Transformation of plants.

A) Tobacco : 10 ml of a overnight culture of Agrobacterium tumefaciens grown under selection was pelleted, the supernatant discarded and the bacteria resuspended in the same volume of an antibiotic free medium. Leaf discs with a size of about $1cm^2$ were bathed in the suspension of the Agrobacteria. Subsequently they were placed on MS-mediuum (after Murashige and Skoog, Physiologia Plantarum 15, 473-497) containing 2% sucrose and 0.8% Bacto-agar. AFter 2 days in the dark at 25°C they were placed on MS-medium containing 100 mg/l kanamycin, 500 mg/l Claforan 1 mg/l benzylaminopurin, 0.2 mg/l naphtyl acetic acid, 2% sucrose and 0.8% Bacto agar. Developing shoots were transferred on hormone free MS-medium containing 2% sucrose and 250 mg/l Claforan. Shoots with developed roots were transferred into soil for further growth.

B) Potato

Transformation of potato was performed according to Rocha-Sosa et al. (EMBO J. 8, 23-29 (1989)).

5. Analysis of genomic DNA of transgenic plants

Isolation of genomic plant DNA was performed according to Rogers and Bendich (Plant Mol. Biol. 5, 69-76).

For DNA-analysis 10-20 μg DNA was digested with suitable restriction enzymes and analysed for specific DNA sequences using the Southern Blotting technique (Maniatis et al., (1982) Molecular Cloning : A Laboratory Manual. CSH Press, CSH, New York).

6. Analysis of RNA from transgenic plants

Total RNA was isolated from plant tissue according to Logemann et al. (Analytical Biochemistry 163, 16-20). Each 50 µg of total RNA were analyzed for specific transcripts using the Northern blot type experiments (Maniatis et al., (1982) Molecular Cloning : A Laboratory Manual. CSH Press, CSH, New York).

7. Extraction of protein from transgenic plants

In order to extract total protein, plant tissue was homogenized in protein extraction buffer (25 mM sodium phosphate pH 7.0; 2 mM sodium bisulfite, 2 mM phenylmethylsulfonylfluoride) in the presence of 0.1% (w/v) insoluble polyvinylpyrrolidon (PVP). The homogenate was filtered through cheesecloth, insoluble components pelleted by centrifugation and the protein concentration in the supernatant determined according to Bradford (Analytical Biochemistry 72, 248-254).

8. Detection of proteins by immunological methods (Western-Blot analysis)

For immunological detection of proteins, total protein extracts were first separated according to size using gel electrophoretic separation in the presence of sodium dodecylsulfate. After the separation, proteins were transferred on a nitrocellulose membrane and subsequently analyzed for specific proteins by first incubating the nitrocellulose filter with an antibody specific for the protein to be detected, afterwards incubating it with a second antibody coupled to alkaline phosphatase and subsequently detecting the alkaline phosphatase activity (Maniatis et al. (1982) Molecular Cloning: A Laboratory Manual. CSH Press. CSH, New York)). The amount of a specific protein present in total extracts was estimated by comparing the intensity of the immunological reaction to known amounts of this protein which had been treated in parallel.

9. Detection of rennilase encoded protease activity

Detection of rennilase encoded activity was performed using total protein extracts from leaves of transgenic tobacco plants expressing the 35s-rennilase chimeric gene following the protocol of Foltmann et al. (Foltmann et al., Analytical Biochemistry 146, 353-360 (1985)).

10. Detection of chymosin encoded protease activity

Detection of chymosin encoded activity was performed using total protein extracts from leaves of transgenic tobacco plants expressing the 35s-chymosin chimeric gene following the protocol of Foltmann et al. (Foltmann et al., Analytical Biochemistry 146, 353-360 (1985)).

Examples

Example 1

Construction of the chimeric gene 35-s-chymosin and its transfer and expression in transgenic plants

The chimeric gene 35s-chymosin is composed of the three fragments A, B and C which contain the following sequences: Fragment A contains the promoter of the 35s RNA of the cauliflower mosaic virus (CaMV) on a fragment encompassing nucleotides 6909 to 7437 of the CaMV (Franck et al. (1980); Cell 21, 285-294) and has been cloned as an Eco RI-Kpn I fragment from the plasmid pDH 51 (Pietrzak et al., 1986; Nucleic Acids Res. 14, 5857-5868) between the Eco RI-Kpn I sites of the plasmid pUC 18.

Fragment C contains the poly-adenylation signal of the gene 3 of the T-DNA of the Ti-plasmid pTi ACH5 (Gielen et al., (1984); EMBO J. 3, 835-846, nucleotides 11749-11939), which has been isolated as a Pvu II-Hind III fragment from the plasmid pAGV 40 (Herrera-Estrella et al., (1983) Nature 303, 209-213) and after adding Sph I linkers to the Pvu II site has been inserted into the Sph I-Hind III sites of the poly-linker of pUC 18. Fragment B comprises a Pst I fragment of the pre-pro-chymosin encoding clone pR 26 encompassing nucleotides

-12 to +1288, which has been ligated in to the Pst I site of the poly-linker of pUC 18.

The chimeric gene 35s-chymosin has subsequently been cloned as a Eco RI-Hind III fragment between the Eco RI-Hind III sites of the polylinker of the binary vector pMPK 110 (Eckes, Dissertation Universität zu Köln, 1983). The size of the total plasmid p35s-chymosin is about 6.8 kb.

Subsequently the gene 35-s-chymosin was introduced in binary vectors and transferred into potato and tobacco plants using Agrobacterium mediated gene transfer techniques. Intact and fertile plants were regenerated from transformed cells.

Results:

- Analysis of the transgenic tobacco and potato plants by Southern type experiments shows the presence of intact and non-rearranged copies of the gene 35-s-chymosin in all plants.

- Analysis of total RNA extracted from leaf tissue of transgenic tobacco and potato plants by a Northern type experiment show the presence of an RNA with a length of about 1700 nucleotides specifically hybridizing to a radioactively labelled chymosin probe which is absent from nontransformed control plants. The amount of this RNA varies between independent transformants.

- Analysis of total soluble protein isolated from leaves of transgenic tobacco plants by a Western type experiment using an antibody specific for chymosin shows the presence of an immunologically cross-reacting protein in transgenic tobacco plants, which is absent from non-transformed control plants. This protein migrates at the same position as authentic mature chymosin. 0.1-0.5% of the total soluble plant protein present in transgenic tobacco plants are represented by chymosin.

Analysis of total soluble protein extracted from transgenic tobacco plants in an agar diffusion test shows the presence of a new protease activity absent from non-transformed control tobacco plants.

These results demonstrate that

- transgenic tobacco and potato plants have been obtained which have been genetically modified by transfer and integration of the chimeric chymosin-coding gene 35-s-chymosin
- as a result of this genetic modification these plants produce an RNA specifically hybridizing with a chymosin probe which is absent in nontransformed control plants
- as a further result of this modification the transgenic plants produce chymosin protein as identified by a chymosin specific antibody. Based on its migration behaviour in denaturing gels this chymosin form is the mature chymosin thus demonstrating the correct processing of the pre-pro-chymosin in transgenic plants. The mature chymosin protein represents about 0.1 - 0.5% of the total soluble protein
- as a further result of this genetic modification a new protease activity is detectable in transgenic plants which is absent from nontransformed plants. This new activity is demonstrated to be the result of the production of mature chymosin in these transgenic plants.

Example 2

Construction of the chimeric gene 35-s-M.miehei aspartic protease and its transfer to and expression in transgenic plants.

The chimeric gene 35s-M.miehei aspartic protease is composed of the three fragments A, B and C which contain the following sequences: Fragment A contains the promoter of the 35s RNA of the cauliflower mosaic virus (CaMV) on a fragment encompassing nucleotides 6909 to 7437 of the CaMV (Franck et al. (1980); Cell 21, 285-294) and has been cloned as an Eco RI-Kpn I fragment from the plasmid pDH 51 (Pietrzak et al., 1986; Nucleic acids Res. 14, 5857-5768) between the Eco RI-Kpn I sites of the plasmid pUC 18. Fragment C contains the polyadenylation signal of the gene 3 of the T-DNA of the Ti-plasmid pTi ACH5 (Gielen et al., (1984); EMBO J. 3, 835-846,

nucleotides 11749-11939), which has been isolated as a Pvu II-Hind III fragment from the plasmid pAGV 40 (Herrera-Estrella et al., (1983) Nature 303, 209-213) and after adding Sph I linkers to the Pvu II site has been inserted into the Sph I-Hind III sites of the poly-linker of pUC 18. Fragment B comprises a BamHI-Xba I fragment of the clone pMT 1222 encoding M.miehei pre-pro aspartic protease encompassing nucleotides -14 to +1399, which has been ligated between the Bam HI and Xba I sites of the poly-linker of pUC 18.

The chimeric gene 35s-M.miehei aspartic protease has subsequently been cloned as a Eco RI-Hind III fragment between the Eco RI and Hind III sites of the polylinker of the binary vector pMPK 110 (Eckes, Dissertation Universität zu Köln, 1983). The size of the total plasmid p35s-M.miehei aspartic protease is about 6.8 kb.

The gene 35-s-M.miehei aspargic protease was introduced in binary vectors and transferred into tobacco and potato plants using Agrobacterium mediated gene transfer techniques. Intact and fertile plants were regenerated from transformed cells.

Results:

- Analysis of the transgenic tobacco and potato plants by Southern type experiments shows the presence of intact and non-rearranged copies of the gene 35-s-M.miehei aspartic protease in all plants (data not shown).

- Analysis of total soluble protein isolated from leaves of transgenic tobacco plants by a Western type experiment using an antibody specific for M.miehei aspartic protease shows the presence of an immunologically cross-reacting protein in all transgenic tobacco plants, which is absent from non-transformed control plants. This protein migrates at the same position as authentic mature M.miehei aspartic protease. 0.1-0.5% of the total soluble plant protein present in transgenic tobacco plants are represented by M.miehei aspartic protease.

Analysis of total soluble protein extracted from transgenic tobacco plants in an agar diffusion test shows the presence of a new protease activity absent from non-transformed control tobacco plants.

These results demonstrate that

- transgenic tobacco plants have been obtained which have been genetically modified by transfer and integration of the chimeric gene 35s-M.miehei aspartic protease
- as a result of this genetic modification these plants produce an RNA specifically hybridizing with a M.miehei aspartic protease probe which RNA is absent in nontransformed control plants
- as a further result of this modification the transgenic plants produce M.miehei aspartic protease protein as identified by an antibody specific for M.miehei aspartic protease. Based on its migration behaviour in denaturing gels this form is the mature protease thus demonstrating the correct processing of the pre-pro-protease in transgenic plants. The mature protein represents about 0.1-0.5% of the total soluble protein
- as a further result of this genetic modification a new protease activity is detectable in transgenic plants which is absent from nontransformed plants. This new activity is demonstrated to be the result of the production of mature M.miehei aspartic protease in these transgenic plants.

Example 3

Construction of the chimeric gene p33-chymosin giving rise to tuber-specific expression of the chymosin protein in transgenic potatoes

A Pst I fragment of the pre-pro-chymosin encoding clone pR26 (comprising nucleotides position -12 to +1288) was supplemented with the promoter region of the class I patatin gene B 33 (containing the Dra I - Dra I fragment encompassing position -1512 to position +14 of the class I patatin gene B 33 (Rocha-Sosa et al., EMBO J. 8 23-29, Patent application P 38 43

627.2)) and given the polyadenylatin signal of the gene 3 of the T-DNA of the Ti-plasmid pTi ACH 5 (cf. Example 1). Subsequently the gene p 33-chymosin is introduced in binary vectors and transferred into potato plants using Agrobacterium mediated gene transfer techniques. Intact and fertile plants are regenerated from transformed cells.

Example 4

Construction of the chimeric gene pl 700-chymosin giving rise to leaf-specific expression of the chymosin protein in transgenic plants

A Pst I fragment of the pre-pro-chymosin encoding clone pR 26 (comprising nucleotides position -12 to +1288) was supplemented with the promoter region of the leaf-specific promoter of the ST-LS1-gene (Stockhaus et al., (1987) Proc. Natl. Acad. Sci. USA 84, 7943-7947). This promoter fragment contains the Eco R I-Mbo II fragment comprising position +1 to +1585 of the published nucleotide sequence (Eckes et al. (1986) Mol. Gen. Genetics 205, 14-22).

At the 3'-end of the chymosin-coding sequence the polyadenylation signal of the gene 3 of the T-DNA of the Ti-plasmid pTi ACH 5 (cf. Example 1) was added. Subsequently the gene pl 700-chymosin is introduced in binary vectors and transferred into potato and tobacco plants using Agrobacterium mediated gene transfer techniques. Intact and fertile plants are regenerated from transformed cells.

CLAIMS

1. A plant cell which comprises an inserted DNA sequence encoding an industrial enzyme which is heterologous to said plant cell, with the exception of enzymes conferring improved growth properties or desirable physical characteristics to living plants producing them.

2. A plant cell according to claim 1, wherein the DNA sequence encoding the enzyme is under the control of a regulatory sequence which directs the expression of the enzyme from the DNA sequence in plant cells and intact plants.

3. A plant cell according to claim 2, wherein said regulatory sequence comprises a promoter capable of directing the transcription of the DNA sequence encoding the enzyme in plants.

4. A plant cell according to claim 3, wherein the promoter is selected from the group consisting of the 35s RNA promoter from cauliflower mosaic virus, the class I patatin gene B 33 promoter, the ST-LS1 gene promoter, promoters conferring seed-specific expression, e.g. the phaseolin promoter, or promoters which are activated on wounding, such as the promoter of the proteinase inhibitor II gene or the wun1 or wun2 genes.

5. A plant cell according to claim 3 or 4, wherein the promoter is operably connected to an enhancer sequence.

6. A plant cell according to claim 5, wherein the enhancer is selected from the group consisting of enhancers from the 5'-upstream region of the 35s RNA of CaMV, the 5'-upstream region of the ST-LS1 gene, the 5'-upstream region of the Cab gene from wheat, the 5'-upstream region of the 1'- and 2'-genes of the T_R-DNA of the Ti plasmid pTi ACH5, the 5'-upstream region of the octopine synthase gene, the 5'-upstream region of the leghemoglobin gene, etc.

7. A plant cell according to claim 2, wherein said regulatory sequence comprises a terminator capable of terminating the transcription of the DNA sequence encoding the enzyme in plants.

8. A plant cell according to claim 7, wherein said terminator is the terminator of the octopine synthase gene of the T-DNA of the Ti-plasmid of Agrobacterium tumefaciens, of the gene 7 of the T-DNA of the Ti plasmid pTiACH5, of the nopaline synthase gene, of the 35s RNA-coding gene from CaMV or from various plant genes, e.g. the ST-LS1 gene, the Cab gene from wheat, class I and class II patatin genes, etc.

9. A plant cell according to any of claims 1-8, wherein the DNA sequence encoding the enzyme is operably connected to a DNA sequence encoding a leader peptide capable of directing the transport of the expressed enzyme to a specific cellular compartment (e.g. vacuoles) or to extracellular space.

10. A plant cell according to claim 9, wherein the leader peptide is selected from the group consisting of the leader peptide of proteinase inhibitor II from potato, the leader peptide and an additional about 100 amino acid fragments of potatin, or the transit peptide of various nucleus-encoded proteins directed into chloroplasts (e.g. from the St-LS1 gene, SS-Rubisco genes, etc.) or into mitochondria (e.g. from the ADP/ATP translocator).

11. A plant cell according to any of claims 1-8, wherein the DNA sequence encoding the enzyme is modified in the 5' non-translated region resulting in enhanced translation of the sequence.

12. A plant cell according to any of claims 1-8, wherein the DNA sequence encoding the enzyme is connected to a second DNA sequence encoding a protein or a fragment thereof in such a way

that expression of said DNA sequences results in the production of a fusion protein.

13. A plant cell according to claim 12, wherein the second DNA sequence encodes patatin or a fragment thereof.

14. A plant cell according to any of claims 1-13, wherein the industrial enzyme is selected from the group consisting of proteases, cellulases, hemicellulases, amylases, lipases, phosphatases, oxidoreductases, glucose isomerase and pectinases.

15. A plant cell according to any of claims 1-13, wherein the industrial enzyme is a heme enzyme.

16. A plant cell according to claim 15, wherein the heme enzyme is an oxidoreductase, such as a peroxidase or haloperoxidase.

17. A plant cell according to claim 16, wherein the peroxidase is a Coprinus sp. peroxidase or a plant peroxidase, such as barley or horseradish peroxidase.

18. A plant cell according to claim 14, wherein the enzyme is derived from a higher eukaryotic organism.

19. A plant cell according to claim 14 or 18, wherein the enzyme is selected from the group consisting of chymosin, Mucor miehei aspartic protease, Humicola, Fusarium or Bacillus cellulases, xylanases or mannanases, papain, chymopapain, trypsin and chymotrypsin.

20. A plant cell according to any of claims 1-19, which is derived from a dicotyledonous plant.

21. A plant cell according to claim 20, wherein the plant cell is a tobacco, potato, tomato, or leguminous plant cell.

22. A transgenic plant which comprises an inserted DNA sequence encoding an industrial enzyme which is heterologous to said plant, with the exception of enzymes conferring improved growth properties or desirable physical characteristics to living plants producing them.

23. A plant according to claim 22, wherein the DNA sequence encoding the enzyme is under the control of a regulatory sequence which directs the expression of the enzyme from the DNA sequence in plant cells and intact plants.

24. A plant according to claim 23, wherein said regulatory sequence comprises a promoter capable of directing the transcription of the DNA sequence encoding the enzyme in plants.

25. A plant according to claim 24, wherein the promoter is selected from the group consisting of the 35s RNA promoter from cauliflower mosaic virus, the class I patatin gene B 33 promoter, the ST-LS1 gene promoter, promoters conferring seed-specific expression, e.g. the phaseolin promoter, or promoters which are activated on wounding, such as the promoter of the proteinase inhibitor II gene or the wun1 or wun2 genes.

26. A plant according to claim 24 or 25, wherein the promoter is operably connected to an enhancer sequence.

27. A plant according to claim 26, wherein the enhancer is selected from the group consisting of enhancers from the 5'-upstream region of the 35s RNA of CaMV, the 5'-upstream region of the ST-LS1 gene, the 5'-upstream region of the Cab gene from wheat, the 5'-upstream region of the 1'- and 2'-genes of the T_R-DNA of the Ti plasmid pTi ACH5, the 5'-upstream region of the octopine synthase gene, the 5'-upstream region of the leghemoglobin gene, etc.

28. A plant according to claim 23, wherein said regulatory sequence comprises a terminator capable of terminating the transcription of the DNA sequence encoding the enzyme in plants.

29. A plant according to claim 28, wherein said terminator is the terminator of the octopine synthase gene of the T-DNA of the Ti-plasmid of Agrobacterium tumefaciens, of the gene 7 of the T-DNA of the Ti plasmid pTiACH5, of the nopaline synthase gene, of the 35s RNA-coding gene from CaMV or from various plant genes, e.g. the ST-LS1 gene, the Cab gene from wheat, class I and class II patatin genes, etc.

30. A plant according to any of claims 22-29, wherein the DNA sequence encoding the enzyme is operably connected to a DNA sequence encoding a leader peptide capable of directing the transport of the expressed enzyme to a specific cellular compartment (e.g. vacuoles) or to extracellular space.

31. A plant according to claim 30, wherein the leader peptide is selected from the group consisting of the leader peptide of proteinase inhibitor II from potato, the leader peptide and an additional about 100 amino acid fragments of patatin, or the transit peptide of various nucleus-encoded proteins directed into chloroplasts (e.g. from the St-LS1 gene, SS-Rubisco genes, etc.) or into mitochondria (e.g. from the ADP/ATP translocator).

32. A plant according to any of claims 22-29, wherein the DNA sequence encoding the enzyme is modified in the 5' non-translated region resulting in enhanced translation of the sequence.

33. A plant according to any of claims 22-29, wherein the DNA sequence encoding the enzyme is connected to a second DNA sequence encoding a protein or a fragment thereof in such a way

that expression of said DNA sequences results in the production of a fusion protein.

34. A plant according to claim 33, wherein the second DNA sequence encodes patatin or a fragment thereof.

35. A plant according to any of claims 22-34, wherein the industrial enzyme is selected from the group consisting of proteases, cellulases, hemicellulases, amylases, lipases, peroxidases, phosphatases, oxidoreductases, glucose isomerase and pectinases.

36. A plant according to any of claims 22-34, wherein the industrial enzyme is a heme enzyme.

37. A plant according to claim 36, wherein the heme enzyme is an oxidoreductase, such as a peroxidase or haloperoxidase.

38. A plant according to claim 37, wherein the peroxidase is a Coprinus sp. peroxidase or a plant peroxidase, such as barley or horseradish peroxidase.

39. A plant according to claim 35, wherein the enzyme is derived from a higher eukaryotic organism.

40. A plant according to claim 35 or 39, wherein the enzyme is selected from the group consisting of chymosin, Mucor miehei aspartic protease, Humicola, Fusarium or Bacillus cellulases, xylanases or mannanases, papain, chymopapain, trypsin, chymotrypsin and feed enzymes.

41. A plant according to any of claims 22-40, which is a dicotyledonous plant.

42. A plant according to claim 41, which is a tobacco, potato, tomato, or leguminous plant.

43. A method of producing an industrial enzyme in a plant, the method comprising cultivating a plant according to any of claims 22-42 or a seed or other propagatable part thereof under conditions conducive to the production of the enzyme, and, if desired, recovering the enzyme from the plant.

INTERNATIONAL SEARCH REPORT

International Application No PCT/DK 91/00194

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC5: C 12 N 5/14, A 01 H 5/00, C 12 N 15/82, 9/00

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
IPC5	C 12 N; A 01 H

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in Fields Searched[8]

SE,DK,FI,NO classes as above

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category *	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
P	WO, A1, 9102066 (MOGEN INTERNATIONAL N.V.) 21 February 1991, see the whole document --	1-43
P,X	WO, A1, 9012876 (AKTIESELSKABET DE DANSKE SPRITFABRIKKER (DANISCO A/S)) 1 November 1990, see for example claim 45 --	1-3,7, 11,14, 20-24, 41-43
X	Dialog Information Services, File 5, BIOSIS, Dialog accession no. 7172918, Piruzyan E S et al.: "Expression of escherichia-coli glucose isomerase gene in transgenic plants", & Dokl akad nauk SSSR 305 (3).1989. 729-731	1-3,7, 11,14, 20-24, 41-43
Y	--	4-6,8-10,12-13,25-35

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance, the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance, the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
4th October 1991	1991 -10- 09
International Searching Authority	**Signature of Authorized Officer**
SWEDISH PATENT OFFICE	Mikael Bergstrand

Form PCT/ISA/210 (second sheet) (January 1985)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
X	Dialog Information Services, File 50, CAB abstracts, Dialog accession no. 1090500, Yusibov, V. M. et al.: "Expression of a bacterial gene for glucose isomerase in tobacco cells", & Pushchino, USSR 1988. 86-90 (3 ref.)	1-3,7, 11,14, 20-24, 41-43
Y		4-6,8-10,12-13,25-35
	--	
X	WO, A1, 9007001 (E.I. DU PONT DE NEMOURS AND COMPANY) 28 June 1990, see the whole document	1-11,14, 20-21, 23-32, 35,41-43
Y		4-6,8-10,25-32
	--	
X	EP, A2, 0356061 (CALGENE, INC.) 28 February 1990, see the whole document	1-11,14, 20-32, 35,41-43
Y		4-6,8-10,25-32
	--	
X	EP, A1, 0319353 (PLANT GENETIC SYSTEMS N.V.) 7 June 1989, see the whole document	1-12,20-33,41-43
	--	
X	EP, A2, 0249676 (AKTIESELSKABET DE DANSKE SUKKERFABRIKKER) 23 December 1987, see the whole document	1-11,20-32,41-43
	--	
X	EP, A2, 0218571 (MONSANTO COMPANY) 15 April 1987, see the whole document	1-11,14, 20-32, 35,41-43
	--	
X	WO, A1, 8700865 (CALGENE, INC.) 12 February 1987, see the whole document	1-11,20-32,41-43
	--	

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
X	AU, B, 7162587 (THE GENERAL HOSPITAL CORPORATION) 28 September 1987, see the whole document --	1-11,20-32,41-43
X	EP, A2, 0189707 (PLANT GENETIC SYSTEMS N.V ET AL.) 6 August 1986, see the whole document --	1-11,20-32,41-43

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO.PCT/DK 91/00194

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the Swedish Patent Office EDP file on 91-08-30 The Swedish Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A1- 9102066	91-02-21	EP-A-	0436003	91-07-10
		NL-A-	8901932	91-02-18
WO-A1- 9012876	90-11-01	AU-D-	5531890	90-11-16
WO-A1- 9007001	90-06-28	AU-D-	4828490	90-07-10
EP-A2- 0356061	90-02-28	AU-D-	4055489	90-03-05
		EP-A-	0382834	90-08-22
		WO-A-	90/01260	90-02-22
EP-A1- 0319353	89-06-07	AU-D-	2811889	89-05-23
		EP-A-	0318341	89-05-31
		JP-T-	2501802	90-06-21
		WO-A-	89/03887	89-05-05
EP-A2- 0249676	87-12-23	AU-B-	599072	90-07-12
		AU-D-	6806587	87-07-30
		DE-A-	3702497	88-04-21
		FR-A-	2603451	88-03-11
		GB-A-B-	2187462	87-09-09
		JP-A-	63022191	88-01-29
		NL-A-	8700207	87-08-17
		SE-A-	8700300	87-07-29
		US-A-	4886753	89-12-12
EP-A2- 0218571	87-04-15	AU-B-	590597	89-11-09
		AU-D-	6091386	87-02-12
		JP-A-	63032488	88-02-12
		US-A-	4940835	90-07-10
WO-A1- 8700865	87-02-12	AU-B-	603063	90-11-08
		AU-D-	6196686	87-03-05
		EP-A-	0233915	87-09-02
		JP-T-	63500425	88-02-18
		US-A-	4956282	90-09-11
AU-B- 7162587	87-09-28	AU-B-	609391	91-05-02
		EP-A-	0239801	87-10-07
		EP-A-	0258410	88-03-09
		JP-T-	63503196	88-11-24
		US-A-	4923720	90-05-08
		WO-A-	87/05327	87-09-11
		ZA-A-	8701352	87-08-12

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO.PCT/DK 91/00194

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the Swedish Patent Office EDP file on 91-08-30
The Swedish Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
EP-A2- 0189707	86-08-06	AU-B-	591087	89-11-30
		AU-D-	5166485	86-07-17
		JP-A-	61224990	86-10-06